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November, 1953

AIC-365

✓ A DIRECT MICROSCOPIC METHOD MODIFIED FOR
ESTIMATION OF MICROORGANISMS IN
CALIFORNIA FROZEN CITRUS CONCENTRATE ✓

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Use of reconstituted, pH 6 juice in the dye solution, drying the slide under infra-red and staining with a particular solution are suggested as effective techniques for direct microscopic examination of citrus products.



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Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

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A DIRECT MICROSCOPIC METHOD MODIFIED FOR ESTIMATION OF MICROORGANISMS IN CALIFORNIA FROZEN CITRUS CONCENTRATE

The need for a rapid method for estimation of microbial content of frozen concentrated orange juice has been expressed by quality-control bacteriologists in the California citrus industry. Since this product must meet certain bacteriological standards, immediate information regarding the microbial population is required by processors.

Plate counts are commonly used for estimations of bacteriological quality of freshly frozen concentrate. The incubation period of 48 hours or longer required to complete this test means that results may not be available in time to avert trouble due to an unpredicted microbial build-up in processing equipment. On the other hand, a successful direct microscopic test gives the needed information within a few minutes and corrective sanitizing action can be taken immediately to eliminate sources of trouble.

Furthermore, information is often needed regarding the microbial population of frozen citrus concentrates which have been held in storage for prolonged periods. It is well known that the count of viable organisms in concentrates decreases during frozen storage. Since improved staining would result in a preparation in which both dead and live organisms are detectable, investigations on direct-count methods, particularly on staining technique, were undertaken.

REVIEW OF METHOD AND PROBLEMS

The first direct method for estimating bacterial numbers in milk was published by Breed in 1911 (2). Since that time many articles have appeared giving modifications of Breed's method for the estimation of microorganisms in milk, as well as adaptations of the method for use on other food products. Wolford (7) published a direct-count method in 1943 for use in estimating the sanitary quality of frozen vegetables. The American Public Health Association (1) tentatively approved a method for the direct microscopic examination of frozen vegetables in 1946. Stevens and Manchester (6) published a method in 1944 for the direct microscopic examination of citrus juices, and recently Hill, Wenzel, and Barreto (4) introduced a method for the examination of frozen concentrated orange juices.

Essentially, these direct-count methods involve the spreading of 0.01 ml. of the material to be examined over a square centimeter of slide area, drying, staining, rinsing off excess stain, drying the stained film, and examining the preparation under an oil-immersion microscope.

In the use of the direct microscopic method for estimations of microorganisms in citrus juices, several difficulties are encountered. The principal one is the occasional tendency for the stained film to run off the slide when the excess stain is removed by rinsing. Stevens and Manchester (6) attempted to remedy this trouble by mixing the juice with a gelatin solution to insure adherence of the film on the slide. Hill, Wenzel, and Barreto (4) used a dilute dye solution which could be dried without danger of overstaining, thus eliminating the need for rinsing off the excess stain and the danger of

losing the film during rinsing. With this latter method the concentrated orange juice is reconstituted to around 12° Brix and 5 ml. of this juice is mixed with 5 ml. of 0.075 percent crystal violet solution. Of this mixture 0.01 ml. is carefully spread over a square centimeter of slide area and the preparation is dried under a heat lamp. The dried film is then coated with a colorless plastic spray from a pressurized container to seal the film as a permanent slide.

Another difficulty with direct-count methods is the degree of contrast obtained between microorganisms, citrus pulp, and background, with different dyes. Gray (3) suggested the use of two parts methylene blue and one part basic fuchsin solution to obtain greater contrast. This technique appears to give good results, but some workers have found that this preparation has a tendency to overstain. In our experience this overstaining can be overcome, without sacrificing contrast, by reduction of the amount of basic fuchsin. By substituting North's (5) aniline oil methylene blue stain for Gray's methanol solution of methylene blue, precipitation of the dye particles on the slide can be avoided. In the use of methylene blue to stain citrus juices, the effect of pH must be considered. The writer has noted that this dye is more effective at pH 5.5-6.5 than at the pH of orange juice, which for California juices is around 3.5. For this reason it is necessary to adjust the juice to pH 5.5-6.0; otherwise certain of the microbial cells will not stain well. Increasing the pH much above this range results in a heavier staining of the orange pulp by the methylene blue. The heavier staining reduces the contrast between organisms and pulp, which makes detection of the organisms more difficult. This observation is in agreement with those of Swisher of the Sunkist Research Department and Kennison of Bireley's Division of General Foods Corporation (personal communications) who have noticed the effect of pH on staining of orange juice with methylene blue.

The writer has found some difficulties in the use of a plastic spray to seal the film on a slide. The spray may be applied too thick to permit sharp focusing of the microscope when the oil immersion objective is used. It is very fatiguing to work with a slide preparation when a sharp focus cannot be obtained. It has been found that slides prepared without a protective plastic coating can be preserved for later reference if desired.

METHOD FOUND MOST SATISFACTORY

1. Orange concentrate is reconstituted to 12° Brix with a 0.7 percent sodium hydroxide solution, and mixed thoroughly. The reaction of the mixture will be in the desired range of pH 5.5 to 6.0 when California juice is used.
2. Mix equal volumes of the reconstituted juice and stain solution in a stoppered tube by inverting 25 times. Vigorous shaking of the tube is not recommended, since this will result in formation of foam, and the solution will be difficult to pipette. The dye solution is prepared by adding 10 ml. of North's aniline oil methylene blue solution and 2 ml. alcoholic basic fuchsin to 88

ml. distilled water. North's stain is prepared by dissolving 3 ml. aniline oil in 10 ml. 95 percent ethanol, adding 1.5 ml. concentrated HCl with stirring, adding 30 ml. saturated alcoholic solution of methylene blue, and then adding 55.5 ml. distilled water. The alcoholic basic fuchsin is prepared by dissolving 1 gram of basic fuchsin in 100 ml. of 95 percent ethanol. The dilute mixed stain solution should be filtered before use and held under refrigeration to prevent the growth of microorganisms. This solution will keep well up to 3 to 4 weeks, but it should be discarded after this period.

3. Using a Breed pipette, transfer 0.01 ml. of this mixture to a clean slide and quickly spread the material over a one-square-centimeter area with an inoculating needle.
4. Dry the slide preparation under an infra-red lamp, taking care not to overheat the slide after drying has been completed. The drying time should not exceed 3 to 4 minutes when a 250-watt heat lamp in a reflector supported about 1-1/4 inches above the slide is used. Prolonged heating will cause the film to turn brown, probably due to caramelization of the sugars.
5. Examine the preparation under oil immersion. Using a microscope with a field 0.16 mm. in diameter, examine 100 fields and count the microbial cells or clumps of cells present. Multiply this number by 10,000 to obtain the estimated count per ml. of reconstituted juice. Multiply this figure by the dilution factor to obtain the estimated count per ml. of concentrate.

The number of fields which should be observed in each preparation will, of course, vary according to the application intended. When used as a control measure in the production of frozen orange concentrate, counting a small number of fields may be found to be satisfactory. Where an indication of the general microbial quality of the product is all that is desired, as few as 25 or 30 fields may be sufficient. If a more accurate estimate is desired the large microscope factor limits the accuracy of the method when applied to low-count juices, unless a sufficiently large number of fields are observed to make certain that representative areas of the preparation are observed.

The staining technique described above will give preparations in which the microbial cells usually appear as dark-blue bodies against a pink to purple background containing the orange pulp. The orange chloroplasts will not be stained, but will appear as irregular orange-colored bodies. The two-color stained preparation is much easier to study under the microscope than one prepared with a single-color stain.

In some instances when stains were made of juice containing actively growing microorganisms, the microbial cells have appeared to be only lightly stained by the methylene blue. This light staining was apparently due to the reduction of methylene blue to the leuco form by the cells. This result gives the staining method a differentiating property, as it gives some degree of distinction between actively respiring and inactive or dead cells.

The direct microscopic method is used in this laboratory to supplement plate-count data on frozen orange concentrates collected from different plants as a part of the coliform investigation now in progress. Whenever possible, initial plate counts of the samples have been obtained at the time the concentrates were collected. In the analysis of these samples it has been found that the direct microscopic count varied from 1.5 to more than 10 times the initial plate count, the latter being made at the time the sample was packed. At least half of the samples having a direct microscopic estimate of more than 10 times the initial plate count consisted of lots of orange concentrate known to have been made from pasteurized feed juice. Concentrates which had been in freezing storage for more than a year and a half still showed a higher count by the direct method than the initial plate count indicated. Plate counts made at the same time as the direct microscopic estimates sometimes showed less than 1 percent of the initial number of microorganisms still detectable by plate counts. Because the direct microscopic estimate will detect dead as well as live cells the method has merit in that it can shed light on the initial bacteriological quality of a sample of concentrate when plate culture tests would not give this type of information.

The direct microscopic estimate also can be used to detect microbial build-ups in the evaporators. Many citrus processors have based their cleanup schedules on the results of hourly microscopic examinations of concentrate taken directly from the evaporators. Since the stains used thus far in this work have been single dye stains, such as methylene blue or crystal violet, the use of the double dye stain recommended herein should prove to be of value to the microscopist because of less eye fatigue and greater contrast in the stained preparation.

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